Cutaneous Blood Flow and Percutaneous Absorption: A Quantitative Analysis Using a Laser Doppler Velocimeter and a Blood Flow Meter

Lynn K. Pershing, Ph.D., Sue Huether, Ph.D., Rebecca L. Conklin, B.S., and Gerald G. Krueger, M.D.
Department of Internal Medicine (Dermatology) (LKP, RLC, GGK), College of Nursing (SH), University of Utah School of Medicine, Salt Lake City, Utah, U.S.A.

Cutaneous blood flow has been directly quantitated in vivo for the first time without animal death utilizing the rat skin sandwich flap. This was accomplished by conducting experiments that made a direct correlation between two instruments: a laser Doppler velocimeter and an electromagnetic blood flow meter. Data demonstrate that the correlation between these two instruments is high and reproducible (r = 0.96) with a small (1.3%) coefficient of variation. Blood flow to skin in the unmanipulated state varies from 0.7 to 1.2 mls/min in an anesthetized rat. Application of the blood flow correlation to the determination of percutaneous absorption of caffeine across human skin and benzoic acid across rat skin demonstrates that assuming cutaneous blood flow is a particular value day to day in any skin type results in an apparent wide range of total compound absorbed across that skin on independent occasions. Utilizing actual blood flow measurements to calculate the amount of chemical absorbed reduces the range of variability in the total amount of chemical absorbed and provides a more accurate knowledge of events occurring during a particular time of the absorption process.

Quantitation of cutaneous blood flow will be useful in physiologic and pharmacologic studies where actual cutaneous blood flow is likely to be important to the processes studied, e.g., delivery of drug to skin, metabolism within the skin, and disposition of drug to blood and skin following topical drug application. *J Invest Dermatol 92:355–359, 1989*

Percutaneous absorption in vivo is a multistep process in which a compound applied to the skin permeates the skin and either remains, in part, in the skin or is absorbed into the local and then the systemic circulation. Previous work in this laboratory [1,2] suggests that cutaneous blood flow is important in the evaluation of chemical absorption across skin in vivo. These data seem at variance with the theory that states blood flow through the skin is normally so high that the rate of chemical absorption would not be expected to be affected by variation in the blood flow over its physiologic range [3]. The lack of an appropriate in vivo model system has allowed this hypothesis to remain unchallenged. Our laboratory has developed a skin sandwich flap, which is created such that it is supplied and drained by a single, accessible artery and vein [4,5]. This model design and new instrumentation, the laser Doppler velocimeter (LDV) and an electromagnetic Blood Flow Meter (BFM), permit, for the first time, an investigation of the influence of cutaneous blood flow on the rate and extent of percutaneous absorption in vivo.

The need for instrumentation that accurately and non-invasively measures blood flow to the skin has been apparent. The LDV has been utilized for monitoring cutaneous blood flow as a non-invasive technology [6,7]. The analog readout of the instrumentation, however, is in millivolts (mV) and therefore, reflects moment-to-moment trends in blood flow in the capillary bed, but not actual volume per time. The BFM measures, in the vessel selected for study, actual blood flow in milliliters per minute (ml/min), as a function of Faraday's first law of magnetic induction. The BFM has been demonstrated to measure blood flow rates in a variety of vessel sizes [8,9] even as small as 0.75 mm vessels [10]. Measurements of blood flow with the BFM has, however, the disadvantage of being invasive. This interferes with the collection of blood samples from the skin sandwich flap during a percutaneous absorption experiment. Simultaneously monitoring blood flow in the skin sandwich flap by these two different instruments has resulted in a correlation that allows conversion of the noninvasive LDV mV data into actual ml/min blood flow. This communication describes the methodology used to measure blood flow to a selected area of skin in a volume per time basis.

Investigators and clinicians utilize the LDV as an objective measure of capillary blood flow. The data described herein confirm the rationale of the approach and provide the means to make an accurate prediction of blood flow in terms that are more readily appreciated: volume per time. The formula used for calculating flux across the skin into blood in vivo demands a blood flow parameter. Heretofore, this has been assumed to be a particular rate that is consistent on a day-to-day basis. Experiments are presented which demonstrate that this assumption results in highly variable extents of total chemical absorption across the same skin on independent occasions, whereas actual blood flow values provide a more reproducible anal-

Manuscript received December 28, 1987; accepted for publication June 29, 1988.

This work was presented at the Western Section Meeting of Society of Investigative Dermatology in 1987.

This work was supported in part by a grant from the Ciba-Geigy Corporation.

Reprint requests to: Lynn K. Pershing, Ph.D., Department of Medicine, Division of Dermatology, University of Utah School of Medicine, Salt Lake City, UT 84132.

Abbreviations:
- BFM: Blood Flow Meter
- CV: coefficient of variation
- IP: intraperitoneal
- LDV: Laser Doppler Velocimeter
- mls/min: milliliters per minute
- mV: millivolts

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ysis. This type of enumeration is critical to a clear understanding of the disposition of chemicals to and from the skin, and hence to a more clear understanding of therapy for and toxicity to the skin.

MATERIALS AND METHODS

Animals Congenitally athymic (nude) rats (180–220 gm) were purchased from the National Cancer Institute (Frederick, MD). Viable rat/rat or human/rat skin sandwich flaps (n = 5) with an isolated but accessible vasculature were generated as a neurovascular island skin sandwich. The flap is generated by first grafting a split thickness human skin graft (0.5 mm) to the subcutaneous surface of a skin flap on the abdomen of a nude rat [4,5]. The skin sandwich flap is then isolated on its arterial and venous vasculature and transferred to the back of the animal via a subcutaneous tunnel and sutured in place. A total period of 5 weeks is required to generate a skin sandwich flap that is ready for experimentation.

Animals were housed in individual sterilized polycarbonate cages with sterilized bedding in a clean double-filtered animal facility isolator (Duo-Flow, Lab Products) which utilizes a 12-h light cycle. Food and water (pH 2.5) were sterilized and allowed ad libitum [5].

Equipment The laser Doppler velocimeter (LDV: 5000; MedPacific Corp, Seattle, WA) utilizes a fiberoptic probe which is placed on the skin surface. The monochromatic light (632.8 nm) produced by its helium-neon laser penetrates the skin to a depth of 1–1.5 mm. It detects blood flow as a function of the Doppler shift of laser light backscattered from red blood cells moving through the skin [6]. The BFM (Blood Flow Computer™, model RC2000), with a 0.5-mm cuff probe was custom manufactured by Micron Medical (Los Angeles, CA). This cuff probe is placed around the vessel directly supplying the skin sandwich flap and quantitates blood flow velocity in ml/min with 0.1 ml/min accuracy using a calibration factor of 2150 and a scale factor of 10, as a function of Faraday's first law of magnetic induction. A rectal thermocouple probe monitors the rat body temperature throughout the experiment, which is maintained at 37 ± 0.5°C with a water circulating heating pad.

Experimental Conditions for the Blood Flow Correlation Study Animals with intact skin sandwich flaps were anesthetized at various times post generation of the skin sandwich flap, with 100 mg/kg KETAJECT (Parke-Davis, Morris Plains, NJ), intraperitoneally (IP), to induce general anesthesia, and 30 mg/kg IP every 45 min to maintain anesthesia throughout a 4–5 h experimental time period. The artery and vein supplying and draining the flap were exposed through an inguinal incision and isolated using microsurgical techniques. Physical manipulation of the vessels induced some vasoconstriction, which decreased to background by filling the inguinal cavity with bacteriostatic saline (USP 0.9%; Elkin-Sinn Inc, Cherry Hills, NJ) for 15 min prior to conducting the experiment. The LDV was attached to the surface of the host side of the skin sandwich flap with a dual adhesive stick (MedPacific, Seattle, WA) and left in place throughout the duration of the experiment. The site chosen for placement of the LDV was identified as a site that had a LDV value which approximated the mean of five random values on that surface of the flap. The 0.5-mm cuff probe of the BFM was attached with fine nosed forceps to the femoral artery, at a position immediate to entry into the sandwich flap. The sandwich flap is constructed such that the only tissue served beyond this position is the flap itself [5]. Maintenance of the cuff probe in one location at 90° to the artery throughout the correlation experiment was necessary to insure proper fit of the cuff probe on the artery and accurate readings. Placement of the noninvasive LDV probe on the surface of the flap and the invasive cuff probe of the BFM on the supplying vessel thus allowed simultaneous monitoring of cutaneous blood flow by two different technologies (Fig 1). Baseline cutaneous blood flow was monitored with both instruments for 10 min to determine the variation of non-manipulated cutaneous blood flow. Zero blood flow was that which occurred following clamping of the artery proximal to the cuff with a microvascular clamp (0.3–1.0 mm vessel size; ASSI, San Diego, CA) for

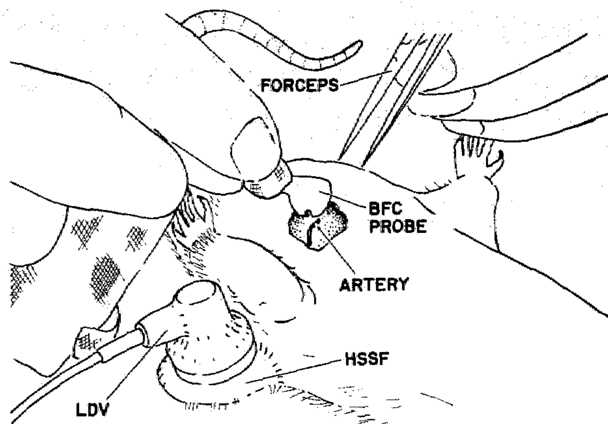

Figure 1. Experimental arrangement for LDV:BFM correlation study. Placement of Laser Doppler Velocimeter (LDV) probe on the human skin sandwich flap (HSSF) and Blood Flow Meter (BFM) probe on the artery nourishing the HSSF.

20 sec. This procedure consistently produced 0 ml/min readout on the BFM and an average of 38.2 mV readout on the LDV within 5 sec of clamping. Following removal of the microvascular clamp, blood flow was monitored with both instruments for 3 min. The hyperemia that usually results from the clamping procedure was followed until blood flow values returned to pre-clamped levels (approximately 3 min post-removal of the microvascular clamp). The clamping procedure was tested 3–4 times on each animal, generating an average of 100+ LDV:BFM measurements from each animal over a 2-h experimental time period. The clamping, releasing, and day-to-day variation resulted in a variety of blood flow values through the artery supplying the flap. The continuous monitoring with the LDV provided nearly instant correlations with the BFM. The inguinal site was sutured with 5.0 black monofilament nylon (ETHILON 5.0 with PS-2 needle; Ethicon, Inc., Somerville, NJ) at the completion of each correlation experiment, and the animals were allowed to fully recover from anesthesia for about 1 h while remaining on a 37°C water circulating heating pad.

Experimental Compounds 1-methyl ^{14}C caffeine (SA = 47.5 mCi/mM) in ethanol and 7-^{14}C benzoic acid (SA = 10.7 mCi/mM) in phosphate buffered saline pH 6.0, were both greater than 98.5% purity and were purchased from New England Nuclear Research Products, Dupont (Boston, MA).

Experimental Protocol for Percutaneous Absorption Experiments The nude rats with a human skin or nude rat skin sandwich flap were anesthetized throughout a 4-h experimental time period with KETAJECT™ as discussed above. ^{14}C caffeine in ethanol (47 μl) was deposited onto the human skin surface in 7-μl aliquots with a variable 10-μl pipet over a 2-min period. The ethanol in each aliquot was allowed to permeate and/or evaporate before subsequent aliquots were applied. ^{14}C benzoic acid in phosphate buffered saline pH 6.0 (400 μl) was dispensed into a 1.0-cm diameter teflon well placed on the rat skin surface with a dual adhesive (3M, St. Paul, MN) and covered with parafilm to prevent evaporation of the solute solution. A total of 18 local and 9 systemic whole blood samples (~40 μl each) were periodically collected over a 4-h experiment. All blood samples were placed in a 25-ml scintillation vial (Beckman Poly Q; Beckman, Irvine, CA) and digested with 200 μl of a 2:1 (v/v) mixture of Isopropyl alcohol and BTS 450 tissue solubilizer (Beckman, Irvine, CA), respectively, in a 40°C water bath for 1 h. The digested samples were then decolorized with 100 μl of 30% Hydrogen peroxide (Baker) and allowed to sit at room temperature (25°C) for 15 min. The vials were then resubmitted to a 40°C water bath for 30 min. Prior liquid scintillation counting, 100 μl of glacial acetic acid (Baker) and 10 ml of Opti-

fluor™ liquid scintillation cocktail (Packard/United Technologies, Downer Grove, IL) were added to the 25 ml scintillation vial and the contents vortexed for 30 sec. Radioactivity within the various blood samples was quantitated with a Liquid Scintillation Counter (Beckman model LS 8100; Beckman, Irvine, CA).

Calculations Determination of total chemical permeation across skin in vitro over time is quantitated by collecting periodic samples from a stirred receiving chamber of either a side-by-side permeation or Franz cell, thus representing an accumulation of the permeating chemical in this chamber over time. Flux in these experiments is calculated according to the equation

$$\text{Flux } (\mu g/cm^2\ hr) = \frac{[C_R - C_0]\ (\mu g/ml) * Vol_R\ (mls)}{SA\ (cm^2) * T_{sampling}\ (hr)} \qquad (1)$$

where C_R and C_0 are the concentration of the chemical in the receiving chamber at the sampling time and time zero, respectively, Vol_R is the total volume of the receiving chamber, SA is the surface area of skin exposed to the chemical, and $T_{sampling}$ is the sampling time. The stirred but non-circulating receiving chamber in the in vitro system provides a sink condition for the permeating chemical, and therefore chemical accumulation is observed over time. The local circulating blood in the present in vivo model is the initial sink of chemical absorption across the skin with the ultimate sink in the systemic circulation. Blood in the skin sandwich flap is circulating, and therefore accumulation of chemical in the local flap blood is not observed over time. The systemic blood eventually returns to the skin sandwich flap as arterial blood. This arterial blood contains chemical previously absorbed. Quantitation of total chemical absorption across the skin sandwich flap therefore requires the local (immediately draining the skin sandwich flap) and systemic blood concentrations of the chemical (C_{local} and C_{sys}, respectively), the instantaneous blood flow to the treated skin site, as well as the surface area of skin exposed to the chemical to calculate instantaneous flux:

$$\text{Flux } (\mu g/hr\ cm^2) = \frac{[C_{local} - C_{sys}](\mu g/ml) * \text{Blood flow } (mls/hr)}{\text{Surface area } (cm^2)}. \qquad (2)$$

The total amount of chemical absorbed across the skin and into the local venous system draining the treated skin site over 4 h is calculated as the area under the flux-time curve similar to the area under the concentration-time curve by the trapezoidal rule [11]. Data are presented as the percent of total chemical applied.

RESULTS AND DISCUSSION

The correlation of cutaneous blood flow by LDV and BFM in the skin sandwich flap is shown in Fig 2. Blood flow in five different animals (each symbol representing a different animal) was analyzed in this correlation study, with each data point plotted representing an average of at least four independent simultaneous readings from the LDV and BFM in a given animal. The data demonstrate a correlation coefficient (r) = 0.9598, with a y intercept of 38.2 mV and a slope of 75.9 mV/(ml/min). This correlation fits over the range of blood flow values observed from 0 to 2.6 ml/min. Blood flow in the skin sandwich flap of an anesthetized rat normally ranges from 0.7 – 1.2 ml/min with the actual value in a particular animal varying on a day-to-day basis (see below). The reproducibility of the correlation with five different animals is excellent with a coefficient of variation (CV) of 1.3%.

The zero blood flow detected by invasive measurements with the BFM corresponds with 38.2 mV on the noninvasive LDV. Our experience with the LDV in this model system has revealed that even animals that have expired for a period of 30 min exhibit a 35 – 40 mV background reading. The deflection of the LDV reading from 0 mV in association with zero blood flow measured with the BFM is likely the result of background turbulent red blood cell velocity following clamping or background reflection within the skin. This is consistent with the observations by Marks et al [12], where freezing the tissue immediately abolished the 20 – 30 mV

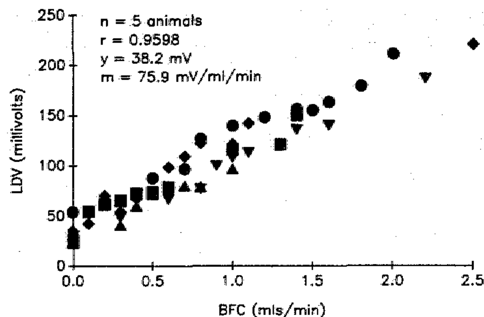

Figure 2. Correlation of cutaneous blood flow as assessed by LDV and BFM. Each symbol represents an individual animal (n = 5 total animals) with each point plotted representing the average of a minimum of four individual readings from the LDV and BFM.

LDV signal observed over 3 h in skin previously removed from a rat.

Analysis of blood flow, as detected by LDV, to the grafted and non-grafted sides of a particular sandwich flap as a function of time after engraftment revealed that both sides of the flap have nearly identical values over a 24-week period [5]. This analysis compared only the blood flow in the capillary beds of both sides of the flap and did not measure actual blood flow to the flap in volume/time (ml/min). Curious, but yet unexplained is the data which show that actual blood flow to a given sandwich flap, as measured with the BFM, varies from day to day (Fig 3). The variability in actual blood flow to the same anesthetized nude rat with a skin sandwich flap over time, with an apparent lack of difference in blood flow to the grafted and non-grafted sides of the sandwich flap, suggests that although temporal variability in overall blood flow to the skin sandwich flap may exist due to experimental and physiologic conditions, the distribution of that blood flow to both sides of the sandwich flap remains relatively constant. The similarity of LDV detected blood flow to grafted and non-grafted rat skin of the sandwich flap confirms that the extent of vascularization of grafted skin is not significantly different from non-grafted skin. This similarity further suggests that LDV values obtained from the human aspect of the rat-human skin sandwich flap can likely be applied to LDV values obtained in situ.

The calculation of chemical flux across skin in this in vivo model (see *Methods*) requires knowledge of blood flow to the local skin site treated with chemical. Concern about the influence of the day-to-day variation in blood flow to the skin sandwich flap on the calculated chemical flux across skin is the result of the present experiments.

The influence of sandwich flap blood flow on the percutaneous absorption of caffeine across human skin following deposition of 36 $\mu g/cm^2$ of ^{14}C caffeine in ethanol onto the same human skin of a human-rat skin sandwich flap on three separate occasions (flap ages 6, 8, and 10 weeks) is presented in Fig 4. The total amount absorbed

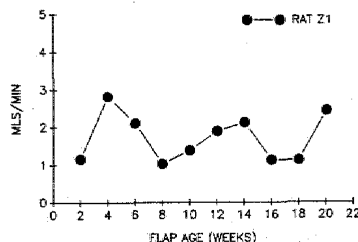

Figure 3. Temporal variation in cutaneous blood flow in a single skin sandwich flap over time.

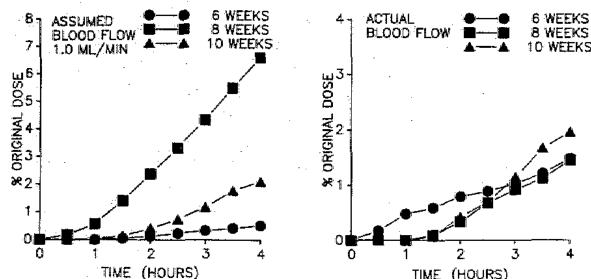

Figure 4. Absorption of ^{14}C caffeine in ethanol across human skin. Total absorption of ^{14}C caffeine in ethanol deposited on human skin calculated with assumed blood flow of 1.0 mls/min *(Left panel)* and actual cutaneous blood flow *(Right panel)* at 6 (●—●), 8 (■—■), and 10 (▲—▲) weeks.

over 4 h is determined using two different approaches, one in which blood flow is assumed to be 1.0 ml/min (average blood flow in the three experiments) in each experiment and the other, in which actual blood flow, as calculated with the LDV:BFM correlation, is utilized. The total amount of ^{14}C caffeine absorbed over 4 h varies dramatically, from 0.5% to 7% of the total amount applied, when blood flow is assumed to be constant at 1.0 ml/min in each experiment (Fig 4, *left panel*). The total amount of ^{14}C caffeine absorbed in the same experiments using actual blood flow values is approximately 1.5%, with significantly less variability (Fig 4, *right panel*).

The influence of temporal blood flow variation in the sandwich flap on total absorption of ^{14}C benzoic acid in phosphate buffered saline pH 6.0 from a 1.0-cm diameter teflon well affixed to the grafted nude rat skin side of a rat-rat skin sandwich flap at various times (flap ages 6, 8, 14, and 16 weeks) is shown in Fig 5. The *left panel* of Fig 5 illustrates the variation in amount absorbed over 4 h when blood flow is assumed to be 1.5 ml/min (average blood flow in the four experiments). The wide fluctuation in values from the same animal over a period of 16 weeks in the *left panel* of Fig 5 becomes dramatically more homogeneous when actual blood flow is utilized to calculate the total amount of ^{14}C benzoic acid absorbed (Fig 5, *right panel*). Despite the fact that there is considerable day-to-day variation in blood flow to the same skin sandwich flap, there is little variation in flap blood flow within a particular experiment. This is likely secondary to the close control of experimental conditions, i.e., monitoring the rat's vital signs, maintaining core temperature at a constant level, and supplying the animal with fluid replacement [5].

The altered flux and total chemical absorbed across the above skin

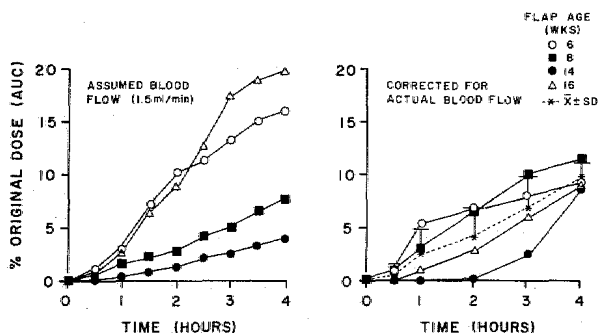

Figure 5. Absorption of ^{14}C benzoic acid in phosphate buffered saline pH 6.0 across a grafted nude rat skin sandwich flap. Total absorption of ^{14}C benzoic acid in phosphate buffered saline (pH 6.0) from a teflon well attached to the skin surface with the assumed blood flow of 1.5 ml/min *(Left panel)* and with actual blood flow *(Right panel)* at 6 (○—○), 8 (■—■), 14 (●—●), and 18 (▲—▲) weeks. Mean ± SD of the 4 experiments (*—*).

sandwich flaps is not the result of changes in the grafted skin itself. Experiments performed with ^{14}C caffeine and ^{14}C benzoic acid across both the grafted and non-grafted aspects of the same nude rat or human skin sandwich flap do not demonstrate any significant differences in the total amount of chemical absorbed over a 4-h period of time (unpublished data). Further, these data confirm that absorption across the grafted skin on separate occasions over a 2-month period have a CV less than 30%.

In conclusion, the data clearly demonstrate that variation in cutaneous blood flow within its physiologic range does have a major effect on the determination of flux and total absorption of two unrelated compounds, caffeine and benzoic acid, across both human and nude rat skin in vivo. Assuming cutaneous blood flow to be constant day to day results in erroneous calculation of percutaneous absorption in vivo. These data support the hypothesis presented by Scheuplein and Bronaugh [3], which states that blood flow rates to the skin within the physiologic range do not significantly alter the flux of a permeating substance across the skin. Confirmation of this theory is demonstrated in the absorption profiles of ^{14}C benzoic acid and ^{14}C caffeine across nude rat and human skin, respectively, in which the total amount of chemical absorbed across either nude rat or human skin on independent occasions utilizing actual blood flow rates collected in independent experiments, reveal very similar profiles of absorption and thus similar flux. Flux, in this in vivo model system, is determined by both the concentration of the permeating chemical in the blood immediately draining the treated skin site and the blood flow to that skin site. Assuming blood flow rates in the skin sandwich flap model to be greater than actual blood flow rates results in an overestimation of flux, while assuming blood flow rates to the skin sandwich flap to be lower than actual blood flow rates results in underestimation of flux and the total amount of chemical absorbed. Under or over estimation of chemical absorption in the skin sandwich flap model therefore results in tremendous variation in the total amount of chemical absorbed as demonstrated in the *left panels* of Figs 4 and 5.

The compounds utilized in the present experiments are both relatively hydrophilic. The degree of influence that cutaneous blood flow may play in the determination of flux and total absorption of more lipophilic compounds in vivo is yet to be demonstrated. Theoretically, cutaneous blood flow is critical in the absorption process of all compounds because evaluation of flux across the skin in the present model necessitates collecting the circulating blood immediately draining the treated skin site. Tissue perfusion is required in vivo to detect and quantitate the absorption process. The influence of local cutaneous blood flow on percutaneous absorption of topically applied compounds is therefore critically important in the understanding of optimum chemical disposition between blood and skin for the treatment of dermatologic diseases, in which it is desirable to maintain high concentrations of the applied compound within the skin, as well as to the pharmacology surrounding transdermal delivery of drugs, i.e., delivery of drugs through the skin into the general systemic circulation for action at distant sites.

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RESEARCH GRANT AVAILABLE IN ALOPECIA AREATA

The National Alopecia Areata Foundation has received an additional substantial donation to support basic alopecia areata research, and/or investigation of potential treatment(s) of alopecia areata. The Ward Family Foundation will award $15,000 for a year's research. This grant is potentially renewable for up to five years or may be awarded annually to the most qualified applicant(s). Applicants are encouraged to present original and innovative proposals.

Requests for applications should be sent to the: National Alopecia Areata Foundation, 714 C Street #216, San Rafael, CA 94901, (415) 456-4644. The deadline for completed 1989 applications is May 1, 1989.